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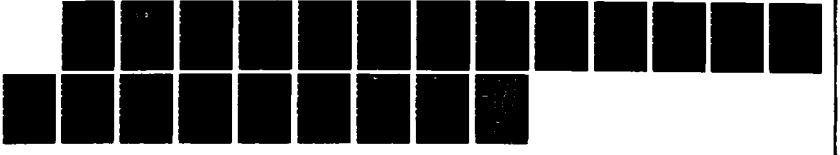

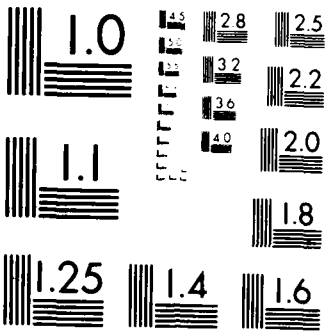
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Research carried out includes sequencing 5S rRNA from Vibrio spp., primarily of marine origin. Analysis of these sequences revealed phylogenetic relationship between these and other members of the family Vibrionaceae. DNA/DNA hybridizations of the Vibrionaceae are also in progress and results will be correlated with 5S rRNA data. In addition, cloning of the 5S rRNA genes from Vibrio natriegens and Vibrio parahaemolyticus into E. coli is underway. Hybridization of apparently non-luminous Vibrio cholerae strains with lux gene probes indicates the presence of luminescence genes which are not being expressed. Sequencing of the chitinase operon of Vibrio vulnificus has continued and is expected to be completed by midyear of 1988. The cloning of chitinase genes of the obligately barophilic bacterium BNL-1 is in progress. Fragments and oligomers derived from chitinolytic determinants of chitinase positive organisms are being developed as probes for estuarine and marine bacteria. The development of a method for simple, rapid isolation of total procaryotic DNA from aquatic habitats has been accomplished. Another method under development will address the problem of monitoring low copy number genes in bacteria in the environment.

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Project Goals:

The objectives of the research are to gain an understanding of the ecology, systematics, and molecular genetics of marine bacteria, crucial to the development of marine biotechnology and its applications. Sub-projects are to (i) clone and sequence ecologically relevant genes from members of the genus Vibrio, including deep-sea species, (ii) examine, by means of DNA homology, relatedness between members of the genus Vibrio; (iii) characterize structure-function relationships between 5S rRNA and the 50S ribosomal subunit to elucidate the role of 5S rRNAs and (iv) clone and sequence genes from deep-sea bacteria.

Recent Accomplishments

Sequence analysis of bacterial 5S ribosomal RNAs was continued, with emphasis on species of the Vibrionaceae. In addition, two other 5S rRNA sequences were generated, one from an antarctic Bacillus sp., the other from an ecologically and economically important commensal of oyster larvae. Improvements were also made to the experimental protocols so that sequencing of 5S rRNA is now a routine procedure. Methods used to sequence the 5S rRNA are described in MacDonell et al. 1987.

The 5S rRNA sequences of V. harveyi ATCC 14126 and V. carchariae, ATCC 35084 were reported previously (MacDonell and Colwell, 1985). The sequences were repeated because recent DNA/DNA hybridization data (J. Farmer pers. comm.) suggested that the two species were identical. Re-examination of the 5S rRNA sequences showed that the two species are, indeed, closely related, but

still can be considered separate species. The 5S rRNA sequences of V. ordalii ATCC 33509 and V. tubiashii ATCC 19105 were also determined, and it is concluded from the data (Pillidge and Colwell, 1988) that both species belong to the recently described genus Listonella (MacDonell and Colwell, 1985). A clear definition of the genus, based on 5S rRNA sequence comparisons and numerical taxonomy, is being done to determine the position of the genus within the family Vibrionaceae.

Colwell and MacDonell (1988) reported the existence of a Vibrio sp. from Antarctic rock samples. This finding was based on analysis of 5S rRNA extracted directly from the rock. In addition, a bacterium was isolated by enrichment of crushed rock samples and its 5S rRNA was sequenced. Analysis of the sequence indicates it is a Bacillus sp. Vibrio sp. were also isolated and these are being sequenced to assess similarity to the 5S rRNA sequence that was obtained by direct extraction from the rock samples.

Exciting results were obtained when determination of the 5S rRNA sequence of a common commensal of oyster larvae was accomplished. This microorganism, designated LST, was identified as a member of the genus Alteromonas (Weiner et al., 2988), based on available taxonomic data. Sequence analysis of the 5S rRNA suggests that LST, along with related strains identified by other investigators as Alteromonas belongs to the genus Shewanella. This genus, proposed by MacDonell and Colwell (1985), comprises certain members of Alteromonas, based on 5S rRNA sequence data. The 5S rRNA sequence obtained from LST further suggests that LST

is related to commensal bacteria of the deep-sea hydrothermal vent bivalve Calyptogena, as well as to bacterial commensals of the American clam Solemya and the marine diatom Odontella. The implication of these intriguing and unexpected findings is that a phylogenetically related set of bacteria is commensal to shellfish of widely different geographic distribution, including the hydrothermal vents of the deep sea.

Work involving cloning of a 5S rRNA gene from Vibrio natriegens ATCC 14048 into Escherichia coli strain in 71-18, using the plasmids pEMBL 8+ and pIBI 24 as vectors, is continuing. V. natriegens DNA may be highly methylated, which would make it a target for enzymatic degradation in E. coli, accounting for the difficulty experienced in obtaining clones of interest. The work has been expanded to include V. parahaemolyticus strain VPS 162-71, originally isolated from fish in Japan (Kaper et al., 1984). We have obtained a library of the organism constructed in E. coli using the plasmid vector pCVD 301 (Datta et al., 1984). A 5S rRNA probe was prepared and used to screen the library for 5S rDNA clones. The 5S rRNA was purified from VPS 162-71, partially digested with S1 nuclease and ligated to 32P-pCp using T4 RNA ligase. The 3' end-labelled 5S rRNA was hybridized to colony blots of the library, prepared on nylon membranes. Preliminary results indicate several possible 5S clones which are now being confirmed.

DNA/DNA homologies using the batch hydroxyapatite method (Brenner et al., 1969) are in progress. DNA from nine Vibrio spp. has been extracted in sufficient quantity and purity. In

the coming year, the first nine Vibrio DNA preparations which have been extracted will be hybridized. Eventually, DNA from eighteen additional Vibrio type strains will be extracted and hybridized. The data will be analyzed by computer, programs for which are now being written. The 5S rRNA and DNA/DNA hybridization data will be compared in order to assess the "noise" in each identification system for marine bacteria and to corroborate the phylogenetic analyses of marine bacteria.

The genes responsible for bioluminescence in Vibrio cholerae biotype albensis have been cloned into the suicide vector pEcoR251. Escherichia coli transformed with this construct acquires the bioluminescent phenotype without the addition of aldehyde, suggesting that all of the genes required for light production are present.

To determine genetic relatedness between V. cholerae biotype albensis and two other bioluminescent species, Vibrio harveyi and Vibrio fischeri, the genes which code for the luciferase enzyme will be sequenced by the enzymatic dideoxy method of Sanger (Sanger et al., 1977). A detailed restriction map of the cloned fragment has been obtained for this purpose. This fragment has been subcloned into three different reading frames of pUC plasmids (pUC8, pUC9, pUC19) using five restriction enzymes found in the multiple cloning sites of these plasmids. The purpose of the subcloning is to obtain the genes which code for the luciferase enzyme in the reading frame required for expression. Two sets of experiments are now underway to determine expression of these

genes. In Vibrio harveyi it has been found that clones containing the luciferase genes luminesce in the presence of exogenous aldehyde (Hastings et al., 1969). Screening of the subclones has not yielded a clone which is luminescent upon addition of aldehyde. This experiment will be repeated using different parameters. Expression of the luciferase genes will also be investigated using an in vitro transcription/translation assay.

The study of environmentally important genes, as well as the tracking of genetically engineered microorganisms (GEMS), often necessitates the ability to monitor the presence of genes in a particular habitat. When studying the genetic fluxes of aquatic habitats, the process of monitoring a specific nucleic acid sequence can be complicated by the species diversity of the habitat, the relative abundance of sequences in the total DNA pool, and the sheer volume of the habitat. We have approached this rather broad and complex problem through three specific areas of research; i) the isolation and characterization of an environmentally important bacterial operon (the chitinase operon of Vibrio vulnificus) as a model system, ii) the development of a method to facilitate the isolation of total procaryotic DNA from water samples, and iii) the development of a system to enhance the sensitivity of nucleic acid probes.

Our initial work with the chitinase operon of V. vulnificus has been reported (Wortman et al., 1986). Since then, research on this operon has largely centered around determination of the complete DNA sequence. We expect the completion of the sequence analysis early in 1988.

In December, 1987, we isolated several thousand bacterial colonies from Chesapeake Bay sediments. We have selected 450 isolates from this group to be probed with fragments of the chitinase operon. The results of the probe experimentation will be compared to results obtained by culturing the isolates on emulsified chitin overlays, and to rapid chemical assays for chitinolytic enzymes (Wortman et al, 1986; O'Brien and Colwell, 1987).

We have recently completed the development of a method for the simple and rapid isolation of total procaryotic DNA from aquatic habitats. The methods are outlined in the appended abstract (Somerville et al, 1988) which has been accepted for presentation at the First International Conference on the Release of Genetically Engineered Microorganisms (REGEM 1). The use of ultrafiltration to concentrate bacterial cells from water samples was chosen due to the difficulty in concentrating "starved" cells via centrifugation (unpublished observation). We have found the method to be simple, relatively rapid, and readily adaptable to shipboard use. We are now in the process of exploring a variety of potential applications for the method, including shotgun cloning of environmental DNA, biomass estimation, enrichment of specific organisms in environmental samples and the tracking of specific genes in aquatic environments.

The method has been used to concentrate 10 to 50 ml samples of hydrothermal vent fluids from both Juan de Fuca (Atlantis II cruise) and 21^{o} N. (provided by Dr. J. Deming) vent sites. Preliminary fluorospectrographic analysis of crude DNA extracts

(Paul and Myers, 1982) of some samples show the presence of significant concentrations of DNA in relatively pure 'black smoker' fluid. This finding, when confirmed, provides important new evidence for the presence of autochthonous bacteria in these environments. We are working to improve our ability to quantify DNA present in relatively unpurified extracts, and to correlate the amount of DNA detected with bacterial numbers. Once the technique has been refined, the remaining black smoker fluid samples will be analyzed.

Chesapeake Bay water was sampled in December, 1987, using our ultrafiltration technique and a method for enrichment culture of enteric bacteria. Results indicate that ultrafiltration, using Sterivex filters, combined with enrichment culture within the filter chamber, is an inexpensive, simple method for culturing bacteria from liter quantities of water. Nucleic acids are also being extracted from Chesapeake Bay water samples and will be probed with synthesized oligomers specific for the chitinase operon of Vibrio vulnificus, using the polymerization-enhanced technique described below. This data will be compared with the results of the probing of the sediment isolates.

The second method under development in the laboratory is intended to address the problems of monitoring genes in the environment which are present in low copy numbers. The method involves probing for the presence of the gene(s) with specific oligomers, and enhancing the probe signal via the polymerization of radiolabeled DNA. As above, the appended abstract (Somerville

et al, 1988a) has been accepted for presentation at REGEM 1. Experimentation to date indicates that an acceptable signal to noise ratio can be attained by 3'-OH blocking procedures established in our laboratory. Current experiments are intended to optimize the polymerization reactions, and to compare signal sensitivity to available technology.

Plans for next year

We plan to conduct the following studies:

1. Resolution of the systematics of bacterial commensals of marine invertebrate organisms, beginning with the confirmed determination of the relationship of LST to Shewanella spp.

2. Further analysis of antarctic rock samples for Vibrio spp. and related microorganisms, and their relationship to species of the Vibrionaceae.

3. Determination of the 5S rRNA sequences of newly described Vibrio spp. e.g. V. hollisae, V. furnissi and V. orientalis, in order to expand knowledge of the Vibrionaceae, a family widely distributed in the world oceans.

4. Continue development of methods for extracting DNA from marine water samples in a form which can be used for DNA probe analysis. These probes will include those derived from the chitinase, luciferase and toxin genes cloned thus far, as well as synthetic oligonucleotide probes.

5. Complete sequence analysis of the *chi* operon in pATW 501 and extrapolate the methodology to cloning of the urease gene.

6. The cloned 5S rRNA gene(s) will be isolated, mapped using restriction endonucleases, and sequenced using the dideoxy method of Sanger (11). The 5S rDNA will then be mutated using site-directed oligonucleotide mutogenesis.

7. Establish a phylogeny based upon DNA/DNA homologies for the family *Vibrionaceae* and compare it with that obtained from 5S rRNA sequences by computer, using Principal Components Analysis and computer graphics for analysis of comparability of the methods.

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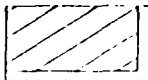

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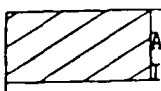

A Simple, Rapid Method for the Direct Isolation of Nucleic Acids from the Aquatic Environment.

C.C.SOMERVILLE, I.T.KNIGHT, W.L.STRAUBE and R.R.COLWELL. Department of Microbiology, University of Maryland, College Park, MD, USA.

The direct isolation of nucleic acids from the environment may be useful in several respects. These include, the estimation of total biomass, detection of specific organisms (e.g. GEMS) and genes, quantitation of gene expression, estimations of species diversity and cloning applications. We have developed a method which facilitates the concentration of microorganisms from aquatic samples and the extraction of their nucleic acids. Liter quantities of sample are first blended to dissociate organisms from particulates, passed through a series of prefilters and concentrated on a single, cylindrical filter membrane (Millipore SVGS01015). Cell lysis and proteolysis are carried out within the filter housing. Crude, high molecular weight nucleic acid solutions are then drawn off the filter. These solutions can be immediately analyzed, concentrated, or purified depending on intended application. The method is both simple and economical and provides DNA/RNA of concentration and purity equal to or exceeding that of published methods.

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Probe-Directed, Polymerization-Enhanced Detection of Specific Gene Sequences in the Environment.

C.C.SOMERVILLE, I.T.KNIGHT, W.L.STRAUBE and R.R.COLWELL. Department of Microbiology, University of Maryland, College Park, MD.

The ability to detect genetically engineered microorganisms (GEMs) in the environment may be considered a necessary prerequisite for their release. In some cases such detection will be complicated by a rapid dilution of the GEMs, both by the shear volume of the habitat and its species diversity. While antibody-mediated detection methods may be sensitive and specific for the original organism released, they do not directly monitor the fate of the engineered gene. We have devised a method to enhance the sensitivity of classic gene-probing technology based upon the use of specific hybridization probes as primers for chain elongation. Target DNA is first treated with DNA pol I and dideoxy-nucleotides to eliminate free 3'-OH residues. Unlabeled probe DNA is then hybridized to target and serves as primer for extensive chain elongation with labeled deoxy-nucleotide(s). Specificity is determined by probe hybridization, while enhanced sensitivity is provided by polymerization into adjacent genes. With this method it should be possible to increase sensitivity by two orders of magnitude or more over conventional methods.

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